INVOLVEMENT OF INTRAMITOCHONDRIAL PROTONS IN REDOX REACTIONS OF CYTOCHROME *a*

V. Yu. ARTZATBANOV, A. A. KONSTANTINOV and V. P. SKULACHEV
A. N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 117234, USSR

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1. Introduction

The energy-conserving function of mitochondrial cytochrome oxidase has been widely believed to consist in the transmembrane transfer of an electron [1–4], as originally proposed by Mitchell [5]. On the other hand, involvement of protons in the cytochrome oxidase reaction was largely neglected (but see [6–8]), although reduction of oxygen to water needs not only 4 e^- but also 4 H^+ ions. We confirmed the important observation [9,10] that E_m of the high-potential heme of cytochrome oxidase was pH dependent [11]. We also found that it was pH inside mitochondria, that was 'felt' by E_m of cytochrome oxidase [11]. Since the high-potential component of cytochrome oxidase was thought at that time to represent heme a_3 [2,12–14], we interpreted our results as evidence for heme a_3 being localized at the inner face of the mitochondrial membrane [11].

Recent revision of the cytochrome oxidase spectral and potentiometric characteristics [15–17] made us re-investigate the problem. The data reported here (see also [18]) indicate that:

(1) Partial redox-linked protonation is characteristic of both cytochromes *a* and a_3 in the high-potential redox transition of cytochrome oxidase.
(2) Cytochrome *a*, known to react with cytochrome *c* at the outer surface of mitochondria, is protonated from the mitochondrial matrix.

The latter result points to a transmembranous location of cytochrome *a* and does not conform to the 'loop scheme' of membrane potential generation by cytochrome oxidase [5]. An alternative mechanism is presented.

Abbreviations: E_m, midpoint redox potential; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate; Mops, morpholinopropanesulfonate; Tris, tris(hydroxymethyl)amino methane

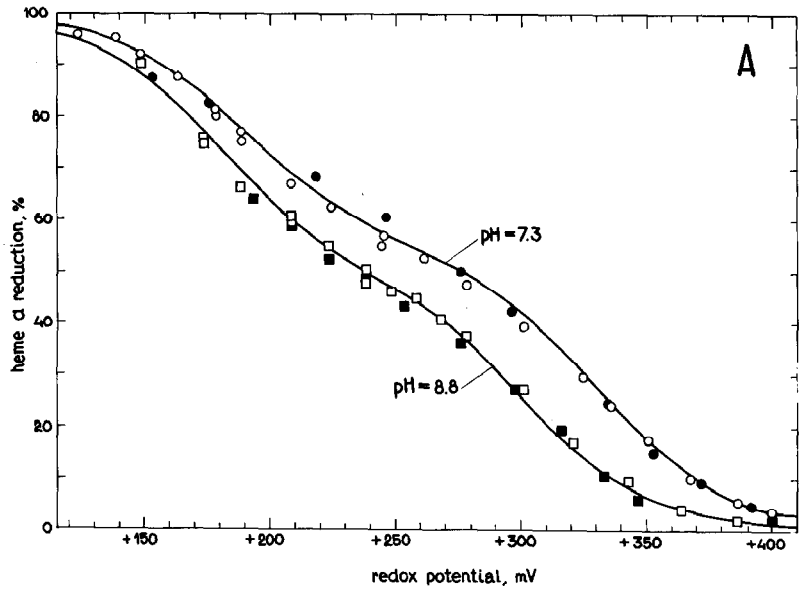
2. Methods

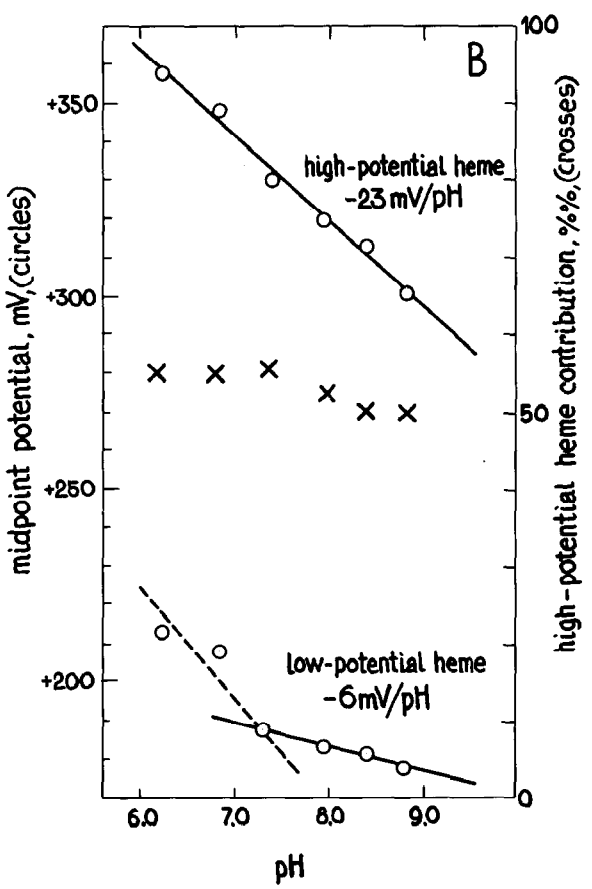
Isolation of rat liver mitochondria and potentiometric titrations of cytochrome oxidase were by conventional procedures [11]. Spectrophotometric measurements were made in a Hitachi-356 instrument. Platinum electrode was calibrated before and after each series of redox titrations with the ferricyanide/ferrocyanide couple using the E_m values given [19].

3. Results and discussion

3.1. *Potentiometric titrations of cytochrome oxidase in anaerobic uninhibited mitochondria;* E_m/*pH relationships*

The results of the anaerobic redox titrations of cytochrome oxidase in uncoupled rat liver mitochondria are given in fig.1. At each pH, experimental points fitted well to the theoretical Nernst curve for oxidoreduction of 2 one-electron components (fig.1A); the E_m values for those components were in good agreement with the data [9,20]. Two points are noteworthy.





1. There is a marked pH-dependence of the high-potential component (–23 mV/pH) while E_m of the low-potential heme is virtually pH-independent above pH ~ 7 (fig.1B). This result confirms the preliminary data in [9, 11] for rat liver mitochondria and is in qualitative agreement with related

Fig.1. Anaerobic potentiometric titrations of mitochondrial cytochrome oxidase at different pH values. Rat liver mitochondria (4 mg protein/ml): 0.2 M sucrose; 50 mM KCl; Mops, Hepes and Tris buffers 20 mM each; 6 μM rotenone; 1.7 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP); 2 μg/ml antimycin. Redox mediators, 0.1 mM diaminodurene, and up to 5 mM ferrocyanide + ferricyanide:
(A) Representative titrations. Open symbols, oxidative titrations with ferricyanide; filled symbols, reductive titrations with ascorbate. Theoretical Nernst curves for 2 one-electron components are drawn through the points with E_m values of +330 and +188 mV at pH 7.3, and +301 and +177 mV at pH 8.8. Contribution of the high-potential heme is 0.55 at pH 7.3 and 0.50 at pH 8.8;
(B) E_m/pH relationships. Each point in fig.1B has been determined from complete reversible potentiometric titration at a given pH. The left-hand ordinate scale is for the E_m values (circles) and the right-hand one is for the ratio of the high and low potential heme contributions to the overall titration curve at 605 nm minus 630 nm (crosses). A possible increase in the E_m/pH-dependence of the low potential heme below pH ~7 is shown by a dashed line (–30 mV/pH).

observations on pigeon heart mitochondria [10]. A somewhat increased E_m/pH dependence of the low potential heme at pH below ~7 (fig.1B) has not been reported previously and is currently being more carefully examined.

2. Contributions of the high- and low-potential components to the overall titration curve at 605 nm minus 630 nm do not appreciably change over the pH range studied (fig.1A,B). Note that, according to the 'neoclassical' model of cytochrome oxidase [15–17], the proportion of high potential heme should greatly decrease or increase with rising pH, if redox-linked protonation were specific either for cytochrome a_3 (as suggested [9,10]) or cytochrome *a*, respectively.

Thus, taking into account recent re-evaluation of cytochrome oxidase spectral and potentiometric properties [17], one can tentatively conclude from the above data that:

(i) Hemes *a* and a_3 have very similar dependences of their midpoint potentials on pH.

(ii) Partial redox-linked protonation of both cytochromes *a* and a_3 manifests itself in the redox transition of cytochrome oxidase from the oxidized to half-reduced state but is faintly visible in the half reduced–reduced transition of the enzyme above pH ~7.

3.2. *The E_m/pH relationships of heme a in cyanide-inhibited mitochondria*

That cytochrome *a* can bind protons upon reduction was further confirmed by the potentiometric titrations of mitochondrial cytochrome oxidase in the presence of cyanide. In the cyanide-inhibited enzyme, it is only cytochrome *a* that undergoes reversible oxidation–reduction, whereas heme a_3 is trapped in the oxidized state. Under these conditions titrations of cytochrome *a* (fig.2A) yielded a single-component Nernst curve with a high E_m (+307 mV at pH 7.4 in agreement with [21,22]) and $n = 0.5$; the reason for this anomalous *n* value (cf. [22]) is at present obscure.

From the two representative titrations shows in fig.2A one can see that E_m of cytochrome *a* becomes more negative by 29 mV as pH is raised from 7.4–8.4. This pH dependency of –30 mV/pH was observed throughout the pH range 6.4–8.9 studied (fig.2B).

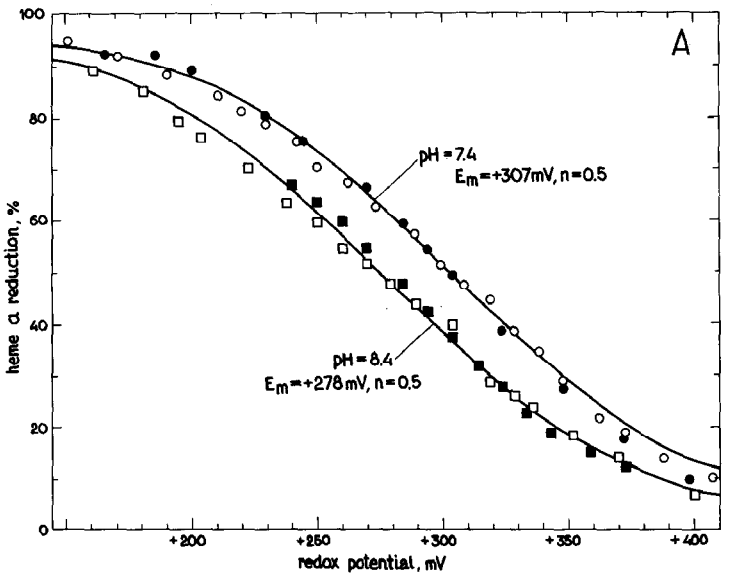
On the other hand, it had been reported [23] that, in carbon monoxide-inhibited rat liver mitochondria (heme a_3 trapped in the reduced state), E_m of cytochrome *a* was pH independent. Comparison of these observations may indicate that ability of cytochrome *a* to bind protons upon reduction depends on the redox state of heme a_3, in accordance with the results given in the previous section.

3.3. *Delayed effect of extramitochondrial pH-jump on the redox equilibrium between cytochrome a and ferrocyanide/ferricyanide couple*

We then attempted to find out whether it was H^+-ion activity inside or outside mitochondria that affected E_m of cytochrome *a*. Cyanide-inhibited mitochondria were poised with the ferrocyanide/ferricyanide redox couple at E_h corresponding approximately to half-reduction of heme *a*. Then KOH or HCl were added so as to change pH in the medium by ~1 unit. Since E_m of $K_4[Fe(CN)_6]$/$K_3[Fe(CN)_6]$ couple is pH independent while that of cytochrome *a* exhibits pH dependency, the system should relax to a new equilibrium state, cytochrome *a* becoming more oxidized or more reduced upon alkalinization or acidification, respectively, at an unchanged E_h of the redox buffer [11,18].

The coupling membrane of intact mitochondria is known to be rather impermeable to H^+ and OH^- ions. Therefore a pH-jump outside mitochondria would be followed by a relatively slow change of the intramitochondrial H^+-ion activity. Were cytochrome *a* in a protonic equilibrium with the internal aqueous phase of mitochondria, its relaxation to a new redox level would lag behind the rapid shift of the external pH, the delay being abolished by protonophorous agents. Alternatively, if redox-linked protonation of cytochrome *a* involved protons from the external medium, one could expect an immediate response of heme *a* to pH-jump outside mitochondria independent of the protonic permeability of the coupling membrane.

The results of such an experiment are shown in fig.3. It can be seen that, in the cyanide-inhibited rat liver mitochondria, both oxidation (traces 1,2) and reduction (traces 4,5) of heme *a* upon a pulse of KOH or HCl are markedly accelerated by a combination of the uncoupler (CCCP) + valinomycin. Valinomycin alone was ineffective (not shown). That electron trans-



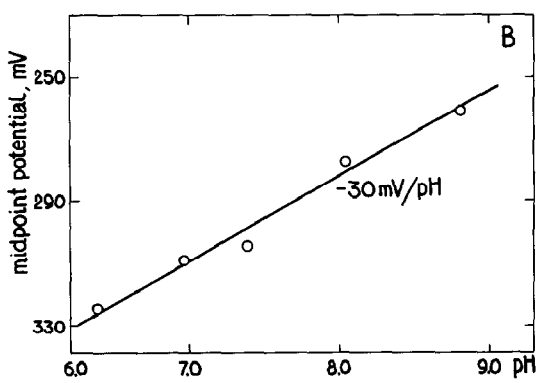


Fig.2. Aerobic potentiometric titrations of cytochrome *a* in cyanide-inhibited mitochondria. Conditions as in the legend to fig.1, but 2 mM cyanide present, and diaminodurene concentration 0.2 mM:
(A) Representative titrations. (●○) pH 7.4; (■□) pH 8.4;
(B) E_m/pH-dependence. Each point has been determined from complete reversible potentiometric titration at a given pH.

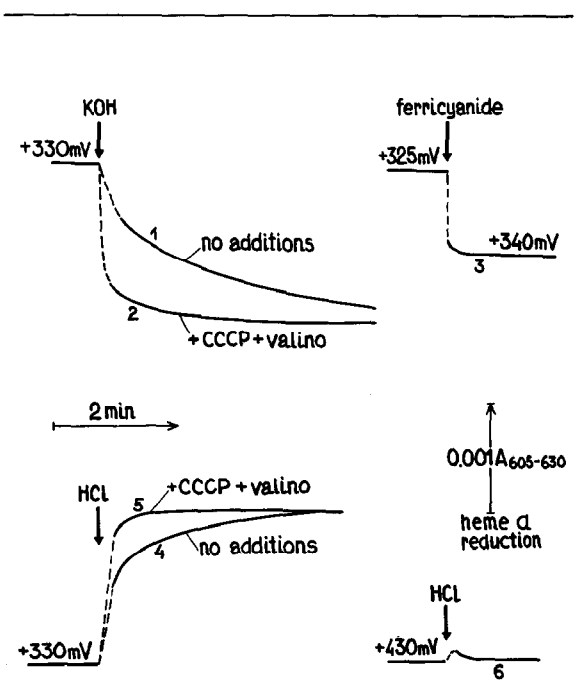


Fig.3. Delayed response of the cytochrome *a* equilibrium reduction level to extramitochondrial pH-jump. Conditions, as in fig.2 but Mops, CCCP and diaminodurene are omitted. 1.7 μM CCCP + 1 μM valinomycin were present in case of traces 2.5 and 6. The KOH additions were calibrated to shift pH from 7.4–8.4; the HCl additions shifted pH from 8.4–7.4. Trace 3, pH 7.4. Redox potential changes were less than ± 5 mV in the acid/base pulse experiments.

fer between cytochrome oxidase and ferro/ferricyanide was not rate-limiting in coupled mitochondria can be seen from the instantaneous oxidation of heme *a* upon addition of ferricyanide (trace 3). No significant changes of absorption at 605 nm minus 630 nm could be observed upon addition of HCl (trace 6) or KOH (not shown) at highly oxidizing or highly reducing

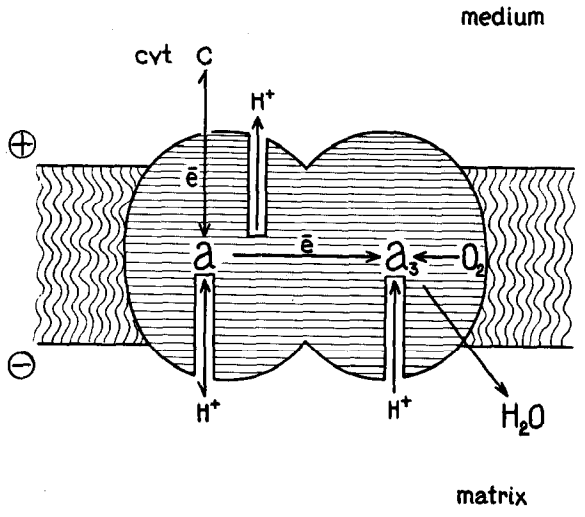
potentials. Similar results were obtained with anaerobic mitochondria in the absence of cyanide [11].

4. Discussion

The simplest explanation for the delayed response of cytochrome *a* to the extramitochondrial pH-jump is that E_m of cytochrome *a* depends on H^+ ion activity inside mitochondria [11,18]. Although the nature of the E_m/pH relationships in cytochrome oxidase remains to be further established, it seems probable that redox-linked protonation of a specific protolytic group in the heme environment is responsible for the effect [10,24,25]. Cytochrome *a* then appears to be located transmembranously, since it oxidizes cytochrome *c* at the outer face of the mitochondria [3,5,26,27] but is protonated from the interior aqueous phase.

Involvement of protons in redox reactions of cytochrome *c* and transmembranous localization of this respiratory carrier cannot be easily reconciled with the 'redox loop' concept of the cytochrome oxidase mechanism [5]. Mitchell's model seems also to be incompatible with [8] where cytochrome oxidase translocates protons across the coupling membrane. On the other hand, there appears to be a possibility of visualizing transmembranous charge transfer by the cytochrome oxidase monomer (cytochrome *a* or a_3), an electron and a proton from the opposite sides of the coupling membrane being accepted by a common active centre of the enzyme (presumably by heme iron and its axial ligand [24,28]).

Based on this principle a new tentative mechanism of membrane potential generation by cytochrome oxidase has been outlined [28] (see also scheme 1); the essential features of this mechanism are cytochromes *a* and a_3 arranged along (not across [5]) the coupling membrane, redox-linked proton transfer reactions across the membrane, and direct involvement of the $a_3 \rightarrow O_2$ reaction step in energy conservation in coupling site III. A model somewhat similar to that presented in scheme 1 was developed independently by Wikström (Proc. 11th FEBS Meet., Copenhagen) on the basis of another line of experiments [6–8,17]. Mention should also be made of some related considerations concerning the role of redox-linked protonation [29,30] and of the 'protonic wells' [31–33], in the mechanism of coupling site II.



Scheme 1 (after [28]). The scheme is a greatly simplified diagramatic visualization of how cytochrome oxidase could operate as an electron–proton membrane potential generator [28] and as a transmembrane proton pump [8]. When cytochrome *a* is in an electronic equilibrium with cytochrome *c*, the heme *a*-linked protolytic group is in a protonic equilibrium with the mitochondrial matrix via the 'entrance proton well' [32]. Cytochrome *a* to come in redox contact with cytochrome a_3, demands a conformational change [34], which brings the heme *a*-linked protolytic group into a protonic equilibrium with the exterior of mitochondria via the 'exit proton well' [32]. Since cyanide is known to block electron transfer from heme *a* to heme a_3, the 'exit proton well' would not have been operative in our experiments with the KCN-inhibited mitochondria (fig.2,3). Starting with the reduced enzyme, heme a_3^{2+} oxidation by O_2 results in the cytochrome a^{2+} increased affinity for H^+-ions and in the protonation of a^{2+} from the matrix. Upon the subsequent oxidation of cytochrome *a* by cytochrome a_3 the protonated heme *a*-linked group releases the H^+-ion into the 'exit' proton well. Cytochrome a_3^{2+} (or a heme-bound reduced oxygen intermediate [28]) is protonated in its turn via the 'entrance' proton well of the a_3 monomer.

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